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(71) Applicant: UTAH STATE UNIVERSITY [US/US]; Suite 120, 1780 North Research Park Way, North Logan, UT 84341 (US).

(72) Inventors: WHITE, Kenneth, L.; 2951 North 1200 East, North Logan, UT 84341 (US). POLEJAEVA, Irina; 380 South 400 West #2, Logan, UT 84321 (US). MORREY, John, D.; 1323 East 1900 North, Logan, UT 84341 (US).

(74) Agents: HOWARTH, Alan, J. et al.; Thorpe, North & Western, L.L.P., Suite 200, 9035 South 700 East, Sandy, UT 84070 (US).

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(54) Title: ESTABLISHMENT, MAINTENANCE, AND TRANSFECTION OF TOTIPOTENT EMBRYONIC STEM CELLS FROM THE EMBRYOS OF DOMESTIC ANIMALS

(57) Abstract

A method is described for establishing embryonic stem (ES) cell lines of domestic animals, such as cattle, sheep, goats, rabbits, and mink. Expanded blastocysts of the domestic animal are treated to remove the zonae pellucidae and then seeded onto a layer of feeder cells in a culture dish containing a suitable medium. The medium comprises plasma that is low in mitogens instead of serum that has undergone the clotting cascade. Addition of leukemia inhibitory factor (LIF), alpha-fetoprotein, and/or hyaluronic acid to the medium also improves the efficiency of establishing such ES cell line. Mink ES cell lines are prepared by pretreating blastocysts with prolactin to reactivate the blastocysts from embryonic diapause. Transfection of such ES cell lines and a composition for use in establishing embryonic stem cell lines are also disclosed.

ESTABLISHMENT, MAINTENANCE, AND TRANSFECTION OF TOTIPOTENT EMBRYONIC STEM CELLS FROM THE EMBRYOS OF DOMESTIC ANIMALS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/007,689, filed November 29, 1995.

BACKGROUND OF THE INVENTION

The present invention relates to animal cell cultures. More particularly, the invention relates to a method of establishing, maintaining, and transfecting totipotent embryonic stem cell lines from domestic animals, such as cattle, sheep, goats, rabbits, and mink.

Embryonic stem (ES) cells, derived from the inner cell mass (ICM) of developing embryos are totipotent, *i.e.* have the ability to develop into any type of cell in the body. Unfortunately, ES cells typically lose totipotency and begin to differentiate aberrantly into other cell types when propagated in cell culture.

Established ES cell lines can be used for rapid propagation of selected domestic animals having valuable or desirable phenotypes. For example, ES cells from embryos from superior animals or transgenic animals can be used to propagate a herd having selected traits within about a year. ES cell lines can also be used to produce transgenic animals at a very high efficiency, up to 100%, whereas the current state-of-the-art with differentiated cells is an efficiency of less than about 5%.

Methods of producing ES cell lines to date have been generally unproductive. Several publications describe ES-like cells from livestock, however, very few have reported the production of viable offspring. *E.g.*, N. Strelchenko, 45 Theriogenology 131-40 (1996).

In view of the foregoing, it will be appreciated that providing a method of efficiently producing ES cell lines from domestic animals such that viable offspring can be produced at high efficiency would be a significant advancement in the art.

BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method of establishing ES cell lines from domestic animals at high efficiency.

It is also an object of the invention to provide a method of establishing and maintaining ES cell lines from domestic animals such that viable animals from such cell lines can be produced at high efficiency.

It is another object of the invention to provide a method of transfecting ES cell lines with a selected gene such that a high proportion of viable transgenic offspring that express the transgene is obtained.

These and other objects are obtained by providing a method of establishing an undifferentiated, pluripotent embryonic stem cell line of a domestic animal comprising the steps of:

(a) obtaining a viable blastocyst, having a zona pellucida, from the animal;

(b) removing the zona pellucida from the blastocyst to obtain an activated blastocyst;

(c) seeding the activated blastocyst onto a feeder layer of cells in a suitable medium, wherein the feeder layer and the medium are capable of supporting the growth of the activated blastocyst and are low in mitogens; and

(d) incubating the seeded blastocyst at a temperature and in an atmosphere such that embryonic stem cells grow and divide.

The domestic animal is preferably a member selected from the group consisting of cattle, sheep, goats, rabbits, and mink. Preferred feeder cells include murine primary embryonic fibroblasts. It is further preferred to pretreat the murine primary embryonic fibroblasts such that mitotic activity thereof is blocked, such as by treatment with mitomycin C. The medium preferably comprises at least about 10% bovine plasma or bovine plasma that has been fractionated to reduce the amount of mitogens therein. It is also preferred to use bovine plasma from a fetal animal. The medium also preferably comprises an effective amount of leukemia inhibitory factor, hyaluronic acid, alpha-fetoprotein, or mixtures thereof. About 1-5 ng/ml of leukemia inhibitory factor, about 10-100 μg/ml of alpha-fetoprotein, and about 100-4000 μg/ml of hyaluronic acid are especially preferred ranges. For establishing mink ES cells, it is also preferred to pretreat the blastocysts with prolactin to stimulate attachment of the blastocysts to the feeder cells.

A method of transfecting an undifferentiated, pluripotent embryonic stem cell line of a domestic animal with a selected gene comprises the steps of:

(a) obtaining a viable blastocyst, having a zona pellucida, from the animal;

(b) removing the zona pellucida from the blastocyst to obtain an activated blastocyst;

(c) seeding the activated blastocyst onto a feeder layer of cells in a suitable medium, wherein the feeder layer and the medium are capable of supporting the growth of the activated blastocyst and are low in mitogens;

(d) incubating the seeded blastocyst at a temperature and in an atmosphere such that embryonic stem cells grow and divide; (e) contacting the growing and dividing embryonic stem cells with an effective amount of a transfection mixture comprising a lipofection reagent and a nucleic acid comprising the selected gene; and

(f) selecting embryonic stem cells that have taken up the nucleic acid comprising the selected gene.

A composition for use in establishing an undifferentiated, pluripotent embryonic stem cell line of a domestic animal comprises at least about 10% of bovine plasma and optionally an effective amount of a member selected from the group consisting of leukemia inhibitory factor, hyaluronic acid, alpha-fetoprotein, and mixtures thereof.

DETAILED DESCRIPTION

Before the present methods for establishing, maintaining, and transfecting ES cell lines are disclosed and described, it is to be understood that this invention is not limited to the particular

configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, reference to a method of establishing an embryonic stem cell line comprising obtaining "a blastocyst" includes references to two or more blastocysts, reference to "a gene" includes reference to one or more of such genes, and reference to "a lipofection reagent" includes reference to two or more of such reagents.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

As used herein, "totipotent" refers to cells that have the ability to develop into any type of cell in the body.

As used herein, "pluripotent" refers to cells that have the ability to develop into any one of several cell types.

As used herein, "serum" means the liquid portion of blood that is prepared after clotting of the blood, *i.e.* after the clotting cascade has been carried out and

other associated processes, such as release of mitogens, has occurred.

As used herein, "plasma" means the liquid portion of blood that is prepared without the blood having gone through the clotting cascade. Plasma is prepared from blood that is treated to inhibit occurrence of the clotting cascade. Plasma can also be fractionated, such as by centrifugation, to remove small amounts of mitogens that can be present despite the clotting cascade and associated processes not having occurred. Such mitogens are believed detrimental to establishment and maintenance of ES cell lines. Plasma should also preferably be free of platelets.

As used herein, "effective amount" means an amount that is nontoxic but sufficient to provide a selected effect and performance. For example, an effective amount of mitomycin C is an amount sufficient to block mitotic activity of feeder cells without being toxic thereto. An effective amount of a protease is an amount sufficient to digest the zona pellucida without being toxic to the blastocyst. An effective amount of leukemia inhibitory factor or hyaluronic acid is an amount sufficient for inhibiting differentiation of embryonic stem cells without being toxic thereto. An effective amount of alpha-fetoprotein is an amount sufficient to enhance growth of embryonic stem cells without being toxic thereto. Further, an effective amount of prolactin is an amount sufficient to reactivate mink blastocysts from embryonic diapause without being toxic thereto. Moreover, an effective amount of a transfection mixture is an amount of

lipofection reagent and nucleic acid sufficient to effect transfection of the nucleic acid into embryonic stem cells without being toxic thereto.

ES cell lines prepared according to the present invention can be used for propagation of domestic animals by methods that are well known in the art. *E.g.*, K.H.S. Campbell et al., Sheep Cloned by Nuclear Transfer from a Cultured Cell Line, 380 Nature 64-66 (1996); K.H.S. Campbell et al., 1 Reviews of Reproduction 40-46 (1996), hereby incorporated by reference. For example, blastocysts from an undesirable animal can be injected with ES cells prepared from a superior animal or a transgenic animal. These injected blastocysts result in chimeric animals, some of which can be germ line chimeras that will contain the gene or genes that are desired to be propagated and selected in resulting offspring. By way of further example, nuclear transplantation technology can be used to propagate domestic animals having the selected genes and/or traits. Oocytes are collected from an undesirable animal and are enucleated by standard methods. The enucleated oocytes are then fused with ES cells from a superior animal or transgenic animal. The fusion results in embryos containing the genome of the superior or transgenic animal. These embryos can then be implanted into suitable recipients for gestation to proceed according to well known methods. *E.g.*, G.E. Seidel, Jr. & S.M. Seidel, The Embryo Transfer Industry, in New Technologies in Animal Breeding 41-77 (B.G. Brackett, G.E. Seidel, Jr., S.M. Seidel eds, 1981), hereby incorporated by reference.

Example 1

The objective of this example is to establish mink ES (MES) cell lines and test them for the presence of alkaline phosphatase (AP), a biochemical marker characteristic of the earliest developmental stages and also of mouse embryonic stem cells. Expanded blastocysts and 4- to 8-cell embryos were flushed from the uteri and oviducts of naturally-mated, wild-type mink. The blastocysts and embryos were then digested with pronase (Sigma Chemical Co., St. Louis, MO; 5 mg/ml at room temperature) to remove the zonae pellucidae and washed in PBS, and the treated embryos were seeded individually into 4-well culture dishes onto feeder layers of mitomycin-C-treated mouse primary embryonic fibroblasts (MPEF). MPEF cells were obtained from 14 day-old murine fetuses after trypsinization. Mitotic activity of MPEF cells was blocked by incubation for 2 hours in 10 μg/ml mitomycin C (Sigma Chemical Co.). Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Logan, Utah) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc.), 0.1 mM 2-mercaptoethanol (Mallinckrodt Chemical Inc., Chesterfield, MO), and 0.1 mM non-essential amino acids (Life Technologies Inc., Gaithersburg, MD) and antibiotics (50-100 IU/ml penicillin, 50 μg/ml streptomycin; Life Technologies, Inc.) was used for embryo culture. The mink reproductive cycle includes a period of embryonic diapause (delayed implantation of 30-40 days). Therefore, blastocysts were kept in suspension for 10 days following removal from the reproductive tract, during which time the blastocysts

remained intact. Then, the blastocysts were "reactivated" by treatment with 5 μg/ml prolactin (PRL; Sigma Chemical Co.). Within 24 hours after addition of prolactin, all blastocysts attached to the feeder layers. Three MES cell lines were obtained from 4 blastocysts. No MES cell lines were established, however, from 4- to 8-cell embryos. All three of these MES cell lines had a high nuclei/cytoplasm ratio, produced simple embryonic bodies, and were positive for AP staining. MES cells differentiated into fibroblast-like and epithelium-like cells when cultured without feeder layers, and these differentiated cells were negative for AP. The MES cell lines were maintained in culture for approximately 5 months without morphological differentiation. These data suggest that PRL stimulates mink blastocyst "reactivation" *in vitro* and that AP activity is a specific and convenient marker for MES cells. The data further suggest that such MES cell lines are pluripotent as judged from their ability to form embryonic bodies and differentiate into several different cell types.

Example 2

In this example, the ability of prolactin to "reactivate" blastocysts and support establishment of MES cells was further investigated. Expanded blastocysts were flushed from the uteri of naturally mated demi minks 13-17 days post-coitum. Pronase was used to remove the zonae pellucidae from embryos, then the embryos were seeded individually into 4-well culture dishes onto feeder layers of mitomycin-C-treated MPEF,

as described in Example 1. The control medium was DMEM supplemented with 10% fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, and antibiotics. Treatment groups were treated with the control medium further containing the following amendments: (1) 0 μg/ml PRL; (2) 5 μg/ml PRL added after 10 days in culture; (3) 10 ng/ml PRL; (4) 1 μg/ml PRL; and (5) 5 μg/ml PRL. The number of MES colonies established by these treatments was observed on days 10, 13, and 18 of cultivation. The results were as follows:

Treatment	No. colonies/No. blastocysts (%)
0 μg/ml PRL	1/7 (14%)
5 μg/ml PRL; 10 days	0/10 (0%)
10 ng/ml PRL	4/10 (40%)
1 μg/ml PRL	6/10 (60%)
5 μg/ml PRL	5/8 (63%)

These results show that treatment of mink blastocysts with about 1-5 μg/ml PRL stimulates blastocyst reactivation and supports establishment of MES cell lines. Established MES cell lines varied in size (8-12 μm in diameter) and had high nuclei to cytoplasm ratios. They further were found to be positive for AP and produced simple embryonic bodies. The MES cell lines prepared according to this example were maintained in culture for 7 weeks without undergoing morphological differentiation.

Example 3

Bovine ES (BES) cell lines were established and tested for AP according to the procedure of Example 1 except that bovine expanded blastocysts were produced by *in vitro* fertilization (IVF) procedures and the

prolactin step was omitted. Such IVF procedures are well known in the art, but will now be briefly described. Ovaries were collected at a local abattoir and transported to the laboratory in 0.9% saline at a consistent temperature of about 25°C. The ovaries were then washed thrice in the same solution before aspiration of oocytes. Oocytes were collected by aspirating small antral follicles (1-7 mm diameter) with a disposable 18-gauge needled connected by glass tubing to a 50 ml conical tube, which served as a trap for collection of the oocytes and follicular fluid. H.W. Hawk & R.J. Wall, Improved Yields of Bovine Blastocysts from In Vitro-Produced Oocytes. I. Selection of Oocytes and Zygotes, 41 Theriogenology 1571-1583 (1994), hereby incorporated by reference. The vacuum pump attached to the trap was set at 150 mm Hg. Only oocytes surrounded by at least three compact layers of cumulus cells and with evenly distributed cytoplasm were selected and randomly assigned to experimental treatments.

Oocytes selected for culture media evaluations were washed three times with HEPES-TALP solution, Parrish et al., Capacitation of Bovine Sperm by Heparin, 38 Bio. Reprod. 1171-1180 (1988), hereby incorporated by reference, and once with maturation medium before *in vitro* maturation (IVM) culture. The *in vitro* maturation of oocytes followed the procedures of Sirard et al., The Culture of Bovine Oocytes to Obtain Developmentally Competent Embryos, 39 Bio. Reprod. 546-552 (1988); Sirard et al., Timing of Nuclear Progression and Protein Synthesis Necessary for Meiotic Maturation of Bovine Oocytes, 40 Bio. Reprod. 1257-1263 (1989), hereby

incorporated by reference. Maturation medium was TCM-199 (Morgan, Morton, Parker, 73 Proc. Soc. of Exp. Biol. Med. 1 (1950); HyClone Laboratories Catalogue, hereby incorporated by reference), 10% fetal bovine serum, 0.5 μg/ml FSH, 5.0 μg/ml LH. IVM culture was for 24 hours in a humidified 5% carbon dioxide atmosphere at 39°C.

Cryopreserved bovine semen from a single bull and the same collection was used (Hoffman A.I. Co., Logan, Utah). Semen used in culture studies met the standard of ≥70% fertilization potential. Straws of frozen semen were thawed in a water bath at 40°C for 1 minute, and the contents of the straw were then transferred to and layered on a 45%-90% Percoll step gradient in a 15 ml Falcon centrifuge tube. Live sperm cells were separated in the Percoll gradient by centrifugation at 700 *g* for 30 minutes. The motile spermatozoa were collected and added to the fertilization medium (Fert-TALP, Parrish et al., *supra*) to provide a final concentration of 1.0 x 10^6/ml. Capacitation was in Fert-TALP containing 25 μg/ml heparin, 0.6% fatty-acid-free bovine serum albumin, without glucose. IVM-cultured oocytes were added to Fert-TALP containing spermatozoa and were cultured for 18 hours in a humidified 5% carbon dioxide atmosphere at 39°C.

At 18 hours after exposure to spermatozoa, the ova were transferred into 15 ml polysytrene tubes containing 1 ml HEPES-TALP supplemented with 0.3% bovine serum albumin. Cumulus and corona cells were removed from the ova by vortexing for 165 seconds. The ova were then placed in 30 μl drops of culture medium (synthetic oviductal fluid; SOF), covered with

dimethylpolysiloxane, and cultured *in vitro* under a humidified 5% oxygen, 90% nitrogen, 5% carbon dioxide atmosphere at 39°C. Embryos that were blastocysts on days 6 and 7 were placed in ES cell culture.

Seven BES cell lines were established from 10 blastocysts. All seven of these BES cell lines had a high nuclei/cytoplasm ratio and produced simple embryonic bodies. BES cell lines were negative for AP activity, however. BES cell lines spontaneously differentiated into neuron-like, epithelium-like, and fibroblast-like cells when not cultured on feeder layers. BES lines were maintained in culture for approximately 5 months without morphological differentiation. These results suggest that BES cell lines prepared according to the present method are pluripotent, but AP activity appears not to be a useful marker for BES cells.

Example 4

ES cells have previously been grown routinely in DMEM supplemented with fetal bovine serum (FBS). It has been found empirically in many laboratories that different lots of serum differ widely in their ability to support ES cell growth. In this example, fractionated bovine plasma (BP) and fetal bovine plasma (FBP), both from HyClone Laboratories, Inc., Logan, Utah, were evaluated as substitutes for FBS for their ability to support the establishment and maintenance of bovine ES cells prepared according to the method of Example 3. Blastocysts were cultured on mouse primary embryonic fibroblast (MPEF) feeder cells, prepared as

described in Example 1, in DMEM supplemented with either 10% FBS, 25% FBS, 25% FBP, 25% BP, or 50% BP. Each medium also contained 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, and antibiotics. The number of blastocysts that attached and formed ES colonies in the various supplemented media were as follows:

Medium	No. colonies/No. blastocysts (%)
10% FBS	10/19 (53%)
25% FBS	12/17 (71%)
25% FBP	21/22 (95%)
25% BP	2/18 (11%)
50% BP	2/16 (12.5%)

These results show that fetal bovine plasma has a significant positive effect on the efficiency of establishing BES cell lines as compared to FBS.

Example 5

In this example, the procedure of Example 4 was followed except that bovine epithelial oviductal cells were used as the feeder layer instead of MPEF cells. None of 27 blastocysts attached to the oviductal cell monolayer. These results show that the selection of cells used as a feeder layer for establishing BES cell lines can have a significant effect thereon. For example, feeder layers of MPEF cells are much superior to bovine epithelial oviductal cells.

Example 6

In this example, the procedure of Example 4 was followed except that blastocysts were cultured over MPEF feeder layer in 25% BP, 10% FBS, or 25% FBP supplemented

as in Example 4 and additionally with 0 ng/ml, 1 ng/ml, 5 ng/ml, or 10 ng/ml of the cytokine, human leukemia inhibitory factor (LIF). It has been reported that LIF can maintain mouse embryonic stem cells in a pluripotent, undifferentiated state. G.B. Anderson, 3 Animal Biotechnol. 165-176 (1992), however, has suggested that a mouse culture system and murine LIF are not effective for preventing differentiation of ES cells from domestic animals. Human LIF is more similar to ovine, and probably bovine, LIF than is mouse LIF. First et al., 6 Reprod. Fertil. Dev. 553-562 (1994). Thus, the efficacy of human LIF, obtained from R&D Systems, Inc. (Minneapolis, MN; lot# BY 1D1), in establishing BES cell lines was investigated in this example. The number of blastocysts that attached and formed ES colonies were as follows:

Medium	LIF (ng/ml)	No. colonies/No. blastocysts (%)
25% BP	0	1/9 (11%)
	1	4/8 (50%)
	5	4/9 (44%)
	10	1/9 (11%)
25% FBP	0	11/11 (100%)
	1	10/10 (100%)
	5	11/11 (100%)
	10	11/11 (100%)
10% FBS	0	9/17 (53%)
	1	13/16 (81%)
	5	11/16 (69%)
	10	8/16 (50%)

The BES lines prepared according to this procedure were maintained in culture for about 7 weeks without morphological differentiation. These results show that about 1-5 ng/ml of human LIF in the culture medium

significantly increases the success rate for establishment of BES cell lines.

Example 7

In this example, the procedure of Example 4 is followed except that blastocysts are cultured over MPEF feeder layer in 25% BP or 25% FBP supplemented as in Example 4 and additionally with 100-4000 μg/ml of hyaluronic acid (Sigma Chemical Co.). Hyaluronic acid is known to inhibit differentiation and enhance proliferation of cells in culture. S. Kato & T. Miyano, Effects of Hyaluronic Acid on the Development of 1- and 2-Cell Porcine Embryos to the Blastocyst Stage in Vitro, 41 Theriogenol. 1299-1305 (1994) (abstract); M.T. Longaker et al., Studies in Fetal Wound Healing: V. A Prolonged Presence of Hyaluronic Acid Characterizes Fetal Wound Fluid, 213 Ann. Surg. 292-96 (1991); R. Stern et al., Studies in Fetal Wound Healing: I. A Factor in Fetal Serum that Stimulated Deposition of Hyaluronic Acid, 24 J. Pediatr. Surg. 789-92 (1989); R. Stern et al., Hyaluronic Acid-Stimulating Activity in the Pathophysiology of Wilms' Tumors, 82 J. Nat'l Cancer Inst. 135-39 (1990); R. Stern et al., Hyaluronic Acid-Stimulating Activity in Sera from Bovine Fetus and from Breast Cancer Patients, 49 Cancer Res. 3499-3505 (1989) (abstract). Hyaluronic has further been shown to sequester or inactivate transforming growth factor-beta (TGF-β), P. Locci et al., Transforming Growth Factor Beta(1)-Hyaluronic Acid Interaction, 281 Cell Tissue Res. 317-24 (1995), which is a differentiation factor in serum or plasma. Hyaluronic acid has also been used to

better cultivate porcine embryos, S. Kato & T. Miyano, Effects of Hyaluronic Acid on the Development of 1- and 2-Cell Porcine Embryos to the Blastocyst Stage in Vitro, 41 Theriogenol. 1299-1305 (1994) (abstract), but has not previously been used to cultivate embryonic stem cells in the presence of platelet-free plasma.

Example 8

In this example, the procedure of Example 4 is followed except that blastocysts are cultured over MPEF feeder layer in 25% BP or 25% FBP supplemented as in Example 4 and additionally with about 10-100 μg/ml of alpha-fetoprotein (Sigma Chemical Co.). Alpha-fetoprotein is found in body fluids of first trimester fetuses, F. Muller et al., First-trimester Amniotic Fluid Acetylcholinesterase Electrophoresis, 9 Prenatal Diagnosis 173-75 (1989), which suggests that this protein is important in embryo cultivation.

Example 9

In this example, efficient transfection of MES cells produced by the procedure of Example 1 was demonstrated. Transfection was with plasmids pGFP-C1 or pGFP-shiva (control) (Clontech, Palo Alto, Calif.). In pGFP-C1, the marker gene, green fluorescent protein (GFP), is under control of the cytomegalovirus immediate early promoter. Three established MES cell lines (passage no. 5) were transfected by lipofection, according to procedures well known in the art, with either the pGFP-C1 or pGFP-shiva plasmids in 35 mm petri dishes. Lipofection reagents were purchased from Life

Technologies (cat. # 18292-0xx). "LIPOFECTIN" Reagent is a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n -trimethylammonium chloride(DOTMA), and dioleoyl phosphotidylethanolamine (DOPE) in membrane filtered water. LIPOFECTIN Reagent interacts spontaneously with DNA to form a lipid-DNA complex. The fusion of the complex with tissue culture cells results in the efficient uptake and expression of the DNA.

MES cells were plated the day before transfection and were 60-70% confluent at the time of transfection. One microgram of plasmid DNA was diluted in 50 μl of serum-free medium. Then, 50 μl of transfection mixture (3 μl of lipofection reagent + 47 μl of serum-free medium) was added to the tube containing plasmid DNA, mixed gently, and incubated for 15 minutes at room temperature. The cells were washed once with 1 ml of serum-free medium and then left in 900 μl of serum-free medium. The lipofection/DNA mixture was added to the dish and gently mixed with the medium. After overnight incubation, 1 ml of medium containing 20% FBS was added and the ES cells were incubated for 36 hours. The medium was then replaced with 1 ml of fresh medium containing 10% FBS. For selection of stable MES colonies, 200 μg/ml of G418 (Life Technologies, Inc.) was added to the medium 72 hours after transfection.

The expanded ES cell pool was used for fluorescence detection. ES cells were washed twice with PBS, harvested with the tip of a Pasteur pipet, placed on a slide, and covered with a glass cover slip. ES cells were observed using a Nikon Diaphot with 10x or 20x

fluorescence objective and a filter set comprising an Omega 405DF40 excitation filter, a 450DRLPO_2 dichroic mirror, and a 510WB40 emission filter. The image was then transmitted via a video camera to a computer loaded with Image-1 software (Universal Imaging), where images were processed, saved, and displayed on the monitor in pseudocolor. The intensity of GFP fluorescence in cells containing pGFP-C1 was significantly greater ($P<0.05$) by Student's T-test as compared to cells containing pGFP-shiva. Transfection efficiencies in MES transfected with pGFP-shiva were as high as 46%, and in MES transfected with pGFP-C1 about 27%. Very low background fluorescence was detected in untransfected cells.

These results indicate that excellent transfection efficiencies can be obtained with ES cell lines prepared according to the present invention. Further, the detection of intracellular GFP apparently does not interfere with cell growth and function, thus GFP is a convenient indicator of gene expression in living MES cells.

Example 10

In this example, the procedure of Example 1 is followed with the exception that the blastocysts are obtained from a rabbit and the prolactin step is omitted.

Example 11

In this example, the procedure of Example 10 is followed with the exception that the blastocysts are obtained from a sheep.

Example 12

In this example, the procedure of Example 10 is followed with the exception that the blastocysts are obtained from a goat.

CLAIMS

We claim:

1. A method of establishing an undifferentiated, pluripotent embryonic stem cell line of a domestic animal comprising the steps of:

(a) obtaining a viable blastocyst, having a zona pellucida, from said animal;

(b) removing the zona pellucida from said blastocyst to obtain an activated blastocyst;

(c) seeding said activated blastocyst onto a feeder layer of cells in a suitable medium, wherein said feeder layer and said medium are capable of supporting growth of said activated blastocyst and are low in mitogens; and

(d) incubating said seeded blastocyst at a temperature and in an atmosphere such that embryonic stem cells grow and divide.

2. The method of claim 1 wherein said domestic animal is a member selected from the group consisting of cattle, sheep, goats, rabbits, and mink.

3. The method of claim 1 wherein said feeder layer of cells comprises murine primary embryonic fibroblasts.

4. The method of claim 3 wherein said murine primary embryonic fibroblasts are pretreated such that mitotic activity thereof is blocked.

5. The method of claim 4 wherein said mitotic activity is blocked by pretreating said murine primary embryonic fibroblasts with an effective amount of mitomycin C.

6. The method of claim 1 wherein said medium comprises at least about 10% bovine plasma.

7. The method of claim 6 wherein said bovine plasma is fractionated to reduce the amount of mitogens therein.

8. The method of claim 6 wherein said bovine plasma is from a fetal animal.

9. The method of claim 1 wherein said medium comprises an effective amount of leukemia inhibitory factor for inhibiting differentiation of said embryonic stem cells.

10. The method of claim 9 wherein said effective amount of leukemia inhibitory factor is about 1-5 ng/ml.

11. The method of claim 10 wherein said leukemia inhibitory factor is of human origin.

12. The method of claim 1 wherein said medium comprises an effective amount of hyaluronic acid for inhibiting differentiation of said embryonic stem cells.

13. The method of claim 12 wherein said effective amount of hyaluronic acid is about 100-4000 μg/ml.

14. The method of claim 1 wherein said medium comprises an effective amount of alpha-fetoprotein for enhancing growth of said embryonic stem cells.

15. The method of claim 14 wherein said effective amount of alpha-fetoprotein is about 10-100 μg/ml.

16. The method of claim 2 wherein said domestic animal is a mink.

17. The method of claim 16 further comprising the step of treating said activated blastocysts with an effective amount of prolactin to stimulate attachment of said activated blastocysts to the feeder layer of cells.

18. The method of claim 17 wherein said effective amount of prolactin is at least about 10 ng/ml.

19. The method of claim 1 wherein said removing said zona pellucida comprises digesting said blastocyst with an effective amount of a protease.

20. The method of claim 19 wherein said protease comprises pronase.

21. A method of transfecting an undifferentiated, pluripotent embryonic stem cell line of a domestic animal with a selected gene comprising the steps of:

(a) obtaining a viable blastocyst, having a zona pellucida, from said animal;

(b) removing the zona pellucida from said blastocyst to obtain an activated blastocyst;

(c) seeding said activated blastocyst onto a feeder layer of cells in a suitable medium, wherein said feeder layer and said medium are capable of supporting the growth of said activated blastocyst and are low in mitogens;

(d) incubating said seeded blastocyst at a temperature and in an atmosphere such that embryonic stem cells grow and divide; (e) contacting said growing and dividing embryonic stem cells with an effective amount of a transfection mixture comprising a lipofection reagent and a nucleic acid comprising said selected gene; and

(f) selecting embryonic stem cells that have taken up said nucleic acid comprising said selected gene.

22. A composition for use in establishing an undifferentiated, pluripotent embryonic stem cell line of a domestic animal comprising at least about 10% of bovine plasma and optionally an effective amount of a member selected from the group consisting of leukemia inhibitory factor, hyaluronic acid, alpha-fetoprotein, and mixtures thereof.

23. The composition of claim 22 wherein said bovine plasma is fractionated to reduce the amount of mitogens therein.

24. The method of claim 23 wherein said bovine plasma is from a fetal animal.

25. The method of claim 22 said effective amount of leukemia inhibitory factor is about 1-5 ng/ml.

26. The method of claim 25 wherein said leukemia inhibitory factor is of human origin.

27. The method of claim 22 wherein said effective amount of hyaluronic acid is about 100-4000 μg/ml.

28. The method of claim 22 wherein said effective amount of alpha-fetoprotein is about 10-100 μg/ml.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18988

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/06, 5/10
US CL :435/240.2, 240.31
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 240.31, 240.1, 240.21, 240.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Polejaeval et al. Isolation and long-term culture of mink and bovine embryonic stem-like cells. Theriogenology, Annual conference of the International Embryo Transfer Society. 08 January 1995, Vol. 43, No. 1, page 300, see entire document.	1-5, 16, 17, 19, 20
X ----- Y	White et al. 'Effect of non-serum supplemented media on establishment and maintenance of bovine embryonic stem-like cells.' Theriogenology, Annual Conference of the IntNo. 1, page 350.	22-25 ------- 1-3, 6-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 FEBRUARY 1997	21 MAR 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	TW for DEBORAH J. R. CLARK Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18988

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Yoshida et al. Gene targetting of DT-diaphorase in mouse embryonic stem cells: Establishment of null mutant and its mitomycin C-resistance. Biochemical and Biophysical Research Communications. 14 September 1995, Vol. 214, No. 2, pages 701-708. See especially page 703, paragraph 1.	22
X ----- Y	Wolf et al. Efficient generation of chimaeric mice using embryonic stem cells after long-term culture in the presence of ciliary neurotrophic factor. Transgenic Research. May 1994, Vol. 3, pages 152-158. See especially pages 152-153.	22, 24 -------- 1, 3, 4, 6, 8-10

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, SCISEARCH, CAPLUS, EMBASE, WPIDS
search terms: embryonic stem cell, culture, feeder layer, activated blastocyst, blastocyst, mitotic activity, mitomycin c, fractionated plasma, leukemia inhibit, hyaluronic acid, alpha-fetoprotein, prolactin, zona pellucida, protease, pronase, transfect, gene, inventor's names